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Efficacy of Multivalent Adenovirus-Based Vaccine against Simian Immunodeficiency Virus Challenge▽

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The prophylactic efficacies of several multivalent replication-incompetent adenovirus serotype 5 (Ad5) vaccines were examined in rhesus macaques using an intrarectal high-dose simian immunodeficiency virus SIVmac239 challenge model. Cohorts of *Mamu-A*01*$^+$*/B*17*$^-$ Indian rhesus macaques were immunized with one of several combinations of Ad5 vectors expressing Gag, Pol, Nef, and Env gp140; for comparison, a *Mamu-A*01*$^+$ cohort was immunized using the Ad5 vector alone. There was no sign of immunological interference between antigens in the immunized animals. In general, expansion of the antigen breadth resulted in more favorable virological outcomes. In particular, the order of efficacy trended as follows: Gag/Pol/Nef/Env ≈ Gag/Pol > Gag ≈ Gag/Pol/Nef > Nef. However, the precision in ranking the vaccines based on the study results may be limited by the cohort size, and as such, may warrant additional testing. The implications of these results in light of the recent discouraging results of the phase IIb study of the trivalent Ad5 HIV-1 vaccine are discussed.

There is a significant body of evidence suggesting that anti-human immunodeficiency virus type 1 (HIV-1) cellular immunity plays a prominent role in controlling viral infection and progression to disease (15, 32, 33). This stimulated substantial research into vaccines capable of eliciting this type of immunity, and several vaccine candidates (5, 6, 8–13, 22, 29–31, 35) have reached various stages of clinical development. However, the viability of this general vaccine approach was recently undermined by the findings in a phase II trial (called the Step Study) that immunization with a replication-defective adenovirus serotype 5 (Ad5) vaccine expressing HIV-1 clade B Gag, Pol, and Nef was not effective in either reducing acquisition rates and/or lowering set point viral loads in infected subjects (2, 25). In fact, more infections were originally observed in the vaccine group than in the placebo arm (2).

The outcomes of the Step Study led to several important questions. Do the results argue against the concept of a HIV-1 vaccine based on the induction of specific T lymphocytes? On the other hand, if cytotoxic T-lymphocyte (CTL) responses are intrinsically valuable for an effective vaccine, what are the shortcomings in the vaccine-induced immunity that contributed to the lack of efficacy in the Step trial? What is the predictive value of preclinical challenge studies for selection of future clinical vaccine candidates? The potential role of CTL responses in an effective vaccine is also challenged by the recently reported phase III study results for the ALVAC vCP1521 prime-AIDSVAX B/E boost vaccine. The efficacy of this vaccine in a low-risk population was recently shown to trend toward prevention of HIV acquisition and not reduction of viral loads (30). Unlike the Step study vaccine, the ALVAC/AIDSVAX vaccine approach utilized a heterologous prime-boost regimen and contained an Env component that may have contributed to the type of outcome observed here. A better understanding of the immune correlates for this vaccine may be possible following further experimental investigations of the samples collected from the phase III study and earlier-stage trials.

Despite the proven efficacy of Ad5 vaccination against simian-human immunodeficiency virus 89.6P (SHIV89.6P) challenge, subsequent primate studies provided equivocal results. In a homologous prime-boost regimen, Ad5 vaccine expressing Gag was ineffective against a high-dose simian immunodeficiency virus SIVmac239 challenge (4, 24). The same study compared this regimen with the DNA prime/Ad5 boost regimen that was found to be efficacious in *Mamu-A*01*$^+$ monkeys; the level of protection in the overall study was correlated with the breadth of epitopes recognized and the frequency of induced antigen-specific CTLs. In this study, we examine whether the expansion of antigens to include Pol, Nef, and Env gp140 using the Ad5/Ad5 regimen would improve the outcome against the same high-dose SIV challenge. Of particular interest is the combination of Gag, Pol, and Nef, for which the homologous human vaccine was utilized in the Step study (29).

MATERIALS AND METHODS

Vaccines. Genes coding for Gag, Pol, Nef, and Env gp140 were synthesized based on codons frequently used in mammalian cells (19). All gene sequences were based on reported sequences from SIVmac239 with the exception of *nef*,

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which was based on the sequence reported for SIVmac251 (18). The *pol* gene consisted only of open reading frames for the reverse transcriptase and integrase domains, for which all active-site acidic residues were replaced with alanines; the *nef* gene contained a substitution of alanine for glycine at position 2 to remove the myristoylation site. Replication-defective Ad5 vectors with E1 deleted expressing each of the SIV genes were constructed following previously established procedures (36).

Immunization and SIV infection. Indian rhesus macaques (*Macaca mulatta*) were typed for major histocompatibility complex type I (MHC-I) allele expression using standard PCR with sequence-specific primers (PCR-SSP) methods (17). *Mamu-A*01*$^+$ animals that coexpressed the B*17 allele were excluded from the study because of the enhanced protective effect of this allelic combination against natural infection (28). Cohorts of 5 *Mamu-A*01*$^+$*/B*17*$^-$ animals were immunized three times (at weeks 0, 4, and 24) with one of the following vaccines: (i) 10^{11} viral particles (vp) of Ad5/SIV Gag and 3×10^{11} vp of a noncoding Ad5 virus; (ii) 10^{11} vp of Ad5/SIV Nef and 3×10^{11} vp of a noncoding Ad5 virus; (iii) 10^{11} vp of Ad5/SIV Gag, 10^{11} vp of Ad5/SIV Pol, and 2×10^{11} vp of a noncoding Ad5 virus; (iv) 10^{11} vp each of Ad5/SIV Gag, Ad5/SIV Pol, Ad5/SIV Nef, and noncoding Ad5 virus; (v) 10^{11} vp each of Ad5/SIV Gag, Ad5/SIV Pol, Ad5/SIV Nef, and Ad5/SIV gp140; and (vi) 4×10^{11} vp of the noncoding Ad5 virus. In all cases, the total vaccine dose was suspended in 1 ml of buffered solution. It should be noted that the doses used in this study were about 10-fold higher than those used in the Step study. The macaques were anesthetized (ketamine-xylazine), and the vaccines were delivered intramuscularly in 0.5-ml aliquots into both deltoid muscles with tuberculin syringes (Becton-Dickinson, Franklin Lakes, NJ). Plasma and peripheral blood mononuclear cell (PBMC) samples were collected following standard protocols.

The SIVmac239 Nef/open virus (28, 39) stock was prepared from plasmid graciously provided by Ronald Desrosiers (Harvard Medical School, Southborough, MA), and the 50% tissue culture infective dose ($TCID_{50}$) was determined via the intrarectal route of administration in rhesus macaques. Each vaccinated animal received 1×10^4 $TCID_{50}$ of the virus intrarectally. All animal care and treatment was in accordance with standards approved by the Institutional Animal Care and Use Committee according to the principles set forth in the *Guide for Care and Use of Laboratory Animals*, Institute of Laboratory Animal Resources, National Research Council. The plasma viral load (VL) was measured by a modified version of the Roche Amplicor UtraSensitive Assay referred to as the SIV UltraSensitive Real-Time PCR Assay, with a lower quantification limit of 50 viral-RNA copies/ml (4). Circulating CD4 levels were determined using Becton Dickinson TruCount tubes (4).

ELISPOT and virus neutralization assays. The gamma interferon (IFN-γ) enzyme-linked immunospot (ELISPOT) assay was conducted following previously described procedures (4). PBMCs, which were shipped overnight to the laboratories at 4°C following collection, were stimulated in the absence (mock) or presence of a defined peptide ($Gag_{181-189}$ CM9) (33) or peptide pool (4 μg/ml per peptide). Pools consisting of 20-amino-acid (aa) peptides overlapping by 10 aa (Synpep, CA) were constructed from entire SIVmac239 Gag and gp140; pools comprised of 15-aa peptides overlapping by 11 aa were prepared for Pol and Nef sequences. The cells were stimulated with the peptides for 20 to 24 h at 37°C in 5% CO_2. Colored spots were counted using an automated counting/microscope instrument and normalized to 1×10^6 PBMCs. A positive response was defined as one having ≥55 spot-forming cells (SFC) per million PBMCs against the stimulatory antigen and ≥3-fold higher than the mock control. SIVmac239 neutralization assays were conducted using CEMX174 human T-lymphoid cells as substrates and following a previously published method (4, 26).

Intracellular flow cytometry staining. PBMCs previously frozen in 90% fetal bovine serum (FBS) and 10% dimethyl sulfoxide (DMSO) freezing medium and stored in liquid nitrogen were quickly thawed in complete RPMI medium supplemented with 10% FBS (HyClone, Logan, UT). PBMCs (1×10^6 per well in 200 μl of complete RPMI medium) were placed in a 96-well U-bottom plate and allowed to rest at 37°C in 5% CO_2 for 4 to 6 h. The cells were then stimulated with 1 μg/ml of each costimulatory antibody (anti-CD28 and anti-CD49d; BD, San Jose, CA), 10 μg/ml of brefeldin A (Sigma); the peptide pools were added to a final concentration of 2 μg/ml per peptide to each sample. The mixtures were incubated overnight (15 to 16 h) at 37°C in 5% CO_2, and 20 μl per well of 20 mM EDTA (mass/volume in 1× phosphate-buffered saline [PBS]) was added to each well for 15 min. The cells were centrifuged at $500 \times g$ for 5 min and washed with fluorescence-activated cell sorter (FACS) buffer (PBS, 1% FBS, 0.01% NaA_3), and stained with surface-staining antibodies—CD8 allophycocyanin (APC)-Cy7 (clone SK1; BD), CD3 PerCPCy5.5 (clone SP23-2; BD), and Violet Amine Reactive Viability Dye (Invitrogen)—for 25 to 30 min. The cells were washed twice with FACS buffer, the supernatant was removed, and the cells were permeabilized with BD Cytofix/Cytoperm solution for 20 min at room temperature. The cells were washed twice with BD Perm/Wash buffer and stained with the intracellular antibodies interleukin 2 (IL-2)-APC (clone MQ1-17H12; BD), tumor necrosis factor (TNF)–phycoerythrin (PE)-Cy7 (clone MAb11; BD), MIP-1β–PE (Clone D21-1351; BD Biosciences), and IFN-γ–fluorescein isothiocyanate (FITC) (clone MD-1; Biosource) for 55 to 60 min. The cells were washed and then fixed with 1% formaldehyde. Samples were analyzed the same day on an LSRII instrument (BD, San Jose, CA). Approximately 300,000 total events were acquired, and the data were analyzed using FlowJo analysis software (Tree Star, Inc.). An electronic gate was drawn around the lymphocyte population, followed by a gate around the viable cells as determined by the Invitrogen dye. Of these, a CD3-versus-CD8 plot was drawn to determine $CD3^+$ $CD8^+$ and $CD3^+$ $CD8^-$ ($CD4^+$ cells). For each T-cell subset, CD4 and CD8, the cells were plotted as histograms showing side scatter light (SSC) on the *y* axis versus each cytokine marker on the *x* axis. A gate was drawn to exclude the cytokine-negative cells. FlowJo software's Boolean gate feature was used to create all the combinations of cytokine populations. Each of these populations was normalized to the number of events per 1 million lymphocytes for the reported final results. A positive response was defined by an antigen-specific response of ≥110 events/10^6 lymphocytes and ≥3-fold above the mock control.

Statistical analysis. Comparisons of immunological or virological parameters were performed by calculating the ratios of the cohort geometric means (GM) and the associated 95% confidence intervals (CI). Correlates of disease protection were determined by associating prechallenge predictor variables and postchallenge VL values. Spearman's rho, a nonparametric and robust rank-based statistical test (14), was used to identify trends of monotonic relationships between VL data and these predictor candidates. All *P* values reported are two-tailed values.

RESULTS

Vaccine-induced immunity. Cohorts of five *Mamu-A*01*$^+$ animals were immunized three times (at weeks 0, 4, and 24) with Ad5 vaccines expressing (i) Gag; (ii) Nef; (iii) Gag and Pol; (iv) Gag, Pol, and Nef; (v) Gag, Pol, Nef, and Env gp140; or (vi) empty vector. Each immunogen was expressed on a separate Ad5 vector, which was given at a 10^{11}-vp dose. The total dose of Ad5 virus given each time was 4×10^{11} vp, and an empty Ad5 vector (no antigen) was used to make up the difference. A higher dose level than that used in the Step clinical study (10^{10} vp per vector) (2) was selected because this preclinical study was primarily intended to show a proof of concept for any immune control and there was no reason at the time to be limited by the known safety profile of the vector in human trials.

Immune responses were measured from PBMCs collected from each animal using the IFN-γ ELISPOT and flow cytometric assays (4). Cytokine secretion was stimulated in the absence (mock) or presence of a defined peptide or peptide pool. Figure 1 shows the ELISPOT data for the individual animals at week 28. The immune responses were highly specific for the vaccines each animal received. Statistical pairwise cohort comparisons of cellular immune responses to each antigen (Gag, Pol, or Nef) gave no indication of immunological interference with any of the antigen-specific immune responses when other vaccine immunogens were added. Gag-specific cellular immune responses were dominated by lymphocytes against the CM9 epitope; the ELISPOT responses against a peptide pool from which the CM9-bearing peptide was excluded were only 9 to 59% of those against the full Gag peptide pool. No anti-SIVmac239 neutralization activity was detected from vaccine serum samples.

PBMCs collected at week 28 from the vaccinees were analyzed using a 7-color intracellular flow cytometric staining assay that detected antigen-specific CD4 and CD8 T cells based on peptide-induced production of IFN-γ, IL-2, TNF-α, and

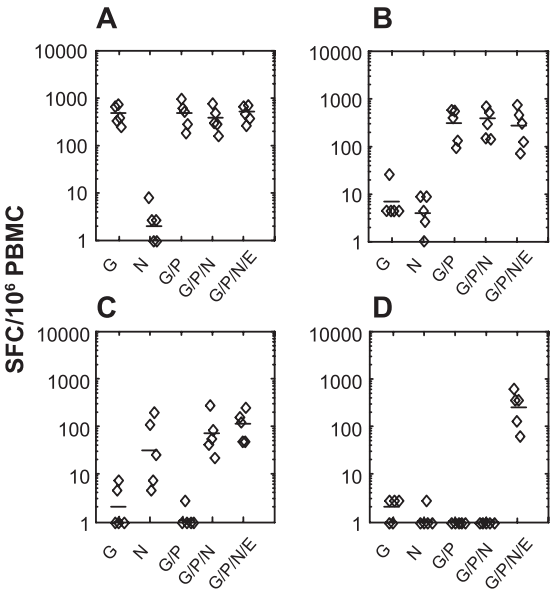

FIG. 1. Levels of circulating T lymphocytes specific for Gag (A), Pol (B), Nef (C), and gp140 (D) for several cohorts listed according to the vaccine received: G, Ad5/SIV Gag; N, Ad5/SIV Nef; G/P, Ad5/SIV Gag plus Ad5/SIV Pol; G/P/N, Ad5/SIV Gag plus Ad5/SIV Pol plus Ad5/SIV Nef; G/P/N/E, Ad5/SIV Gag plus Ad5/SIV Pol plus Ad5/SIV Nef plus Ad5/SIV Env. Shown are the individual animal results (diamonds) and the geometric mean values for the cohort (horizontal lines).

MIP-1β. The results are shown in Fig. 2. Overall, the two most commonly detected antigen-specific CD8 T cells were (i) those of the type which simultaneously produces IFN-γ, TNF-α, and MIP-1β (IFN-γ^+/IL-2^-/MIP-$1\beta^+$/TNF-α^+) and (ii) the IFN-γ^+/IL-2^-/MIP-$1\beta^+$/TNF-α^- type. CD8 cells with these two expression profiles were detected for at least 1 protein antigen in 15 (60%) and 17 out of 25 vaccinees (68%), respectively. A few animals (7 of 25 recipients [28%]) had detectable CD8 cells of the IFN-γ^+/IL-2^+/TNF-α^+/MIP-$1\beta^+$ phenotype for at least one of the vaccine antigens. Multifunctional T cells, particularly those expressing IL-2, have been detected in HIV-1 long-term nonprogressors (1) and have been associated with control of other human pathogens (34). The most and the least commonly detected CD8 responses using this assay were to Gag (17 of 20 [85%] that received the Gag vaccine by itself or in combination) and Nef (6 of 15 [40%]), respectively. The overall functional profiles of the CD8 cells in the vaccinated macaques were very similar to those observed in human recipients of the Ad5 HIV-1 vaccines (7). However, there were minimal $CD4^+$ responses detected among the vaccine recipients using the multicolor flow cytometry method. We note that CD8 responses were rather poorly detected in the Gag/Pol/Nef cohort compared to the other Gag^+ cohorts (Fig. 2) despite the fact that the animals in the former mounted IFN-γ ELISPOT responses to the CM9 epitope that were as vigorous as those of the other Gag^+ cohorts. We cannot rule out the

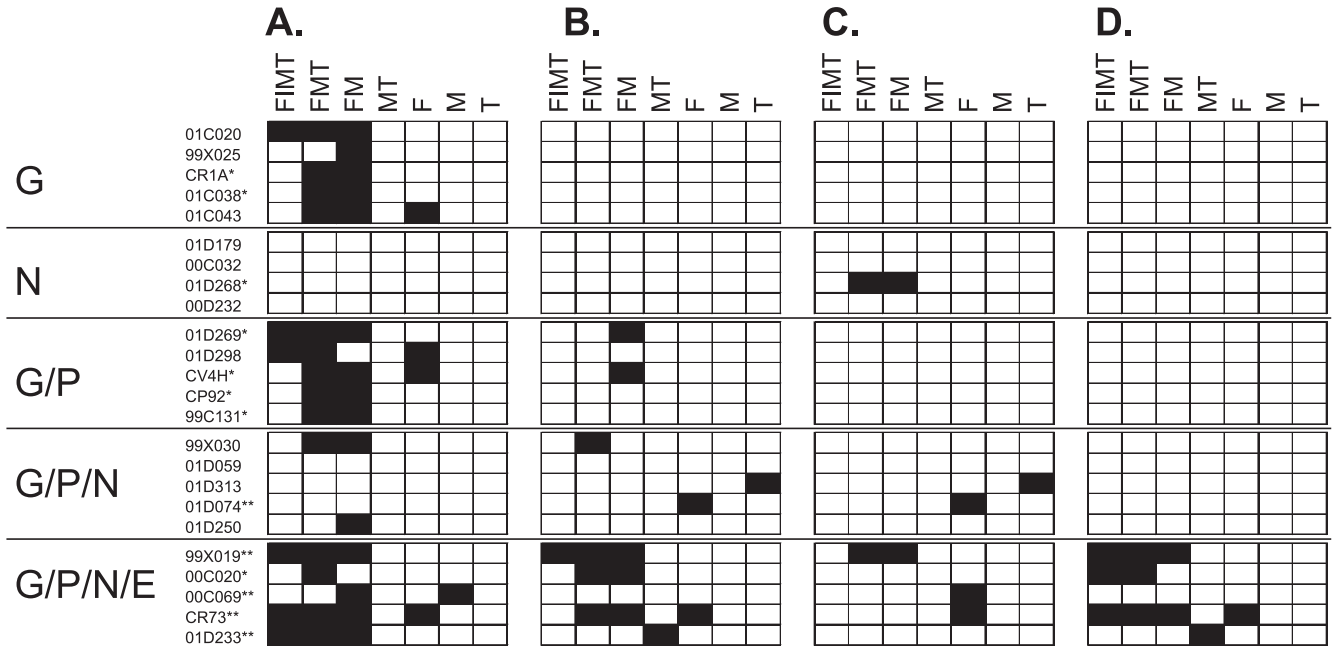

FIG. 2. Expression profiles of CD8 T cells specific for Gag (A), Pol (B), Nef (C), and Env gp140 (D) for each animal (across a row) in the study. The immunogens in the vaccines used are listed in the left column (G, Gag; N, Nef; G/P, Gag/Pol; G/P/N, Gag/Pol/Nef; G/P/N/E, Gag/Pol/Nef/Env). PBMCs stimulated overnight with specific peptide pools were stained for intracellular detection of IFN-γ, IL-2, MIP-1β, and TNF-α. One of the Nef vaccines, 99C105, was not assayed because of very poor cell recovery (not shown). The abbreviations for the markers are as follows: F, IFN-γ; I, IL-2; M, MIP-1β; T, TNF-α. In the top row are various T-cell subsets based on the markers for which the antigen-specific T cells were positive; a marker was specifically not included if those T cells were negative for them. For example, FIMT refers to the subpopulation of T cells that were IFN-γ^+/IL-2^+/MIP-$1\beta^+$/TNF-α^+; FMT, IFN-γ^+/IL-2^-/MIP-$1\beta^+$/TNF-α^+; FM, IFN-γ^+/IL-2^-/MIP-$1\beta^+$/TNF-α^-; F, IFN-γ^+/IL-2^-/MIP-$1\beta^-$/TNF-α^-. For simplicity, those subsets (i.e., IMT, FIM, FIT, FI, IM, FT, IT, and I) that were not observed in any of the samples are not included. A box is shaded if the PBMCs for the macaque were positive for the given T-cell subset, i.e., the response level was above the positivity cutoff (see Materials and Methods), Asterisks mark those animals that resolved their viremia, and double asterisks mark those that controlled their viremia to stable levels during the course of the year of postchallenge monitoring.

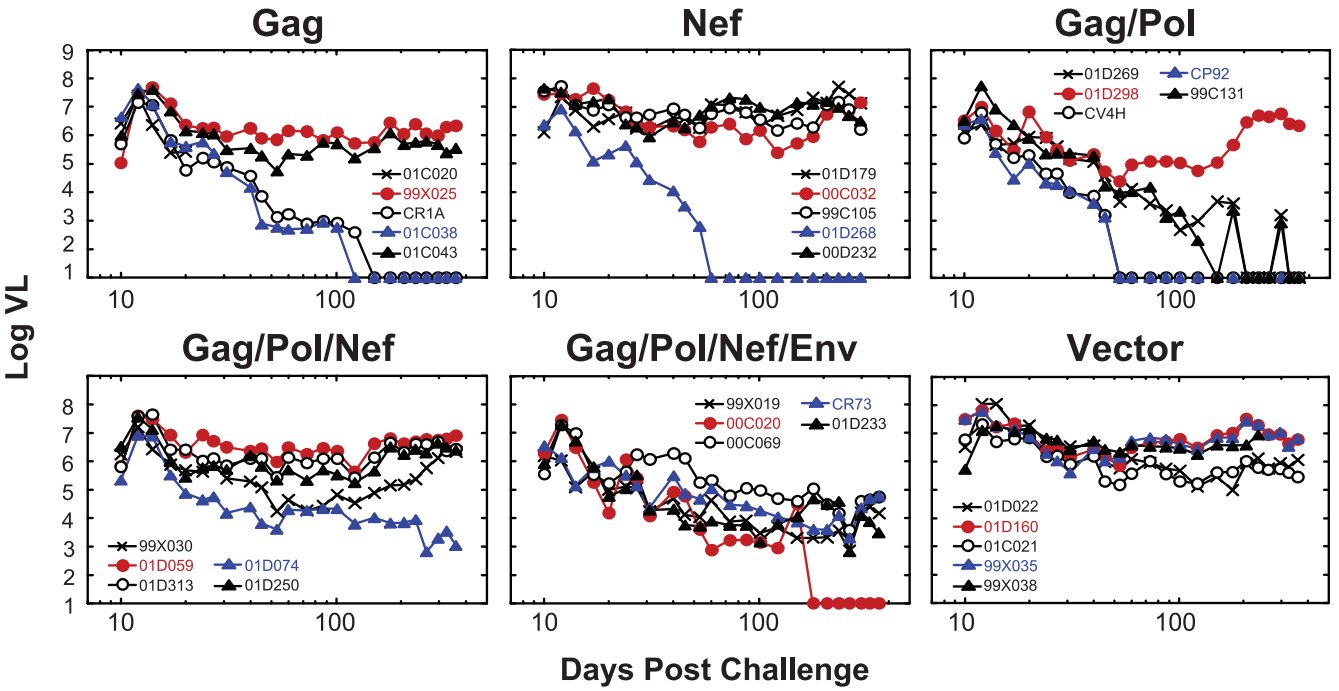

FIG. 3. Plasma viral loads (copies/ml) as a function of time after challenge (log scale) for all of the animals in the study. The legends within each panel represent the tags for the animals within the group; above each graph is the immunogen(s) contained in the vaccine.

possibility that the poor detection levels were due to differential effects of prolonged frozen storage on the integrity of the PBMCs used for the analyses; in contrast, input cells for the ELISPOT assay were from freshly collected samples.

Viremia and CD4 counts following viral challenge. At 10 weeks after the third dose, each animal was given 1×10^4 $TCID_{50}$ of the SIVmac239 Nef/open virus intrarectally; the plasma VL (Fig. 3) and circulating CD4 levels were determined over a period of 360 days. Peak VL was observed between 10 and 14 days for all the animals. The individual peak VLs and the geometric mean values for the various cohorts are shown in Fig. 4A. The control animals exhibited peak VLs ranging from 1.93×10^7 to 1.11×10^8, with a geometric mean of 4.45×10^7 copies/ml. The geometric means of the peak VLs for the Gag/Pol/Nef/Env and Gag/Pol vaccine cohorts were 4.9-fold (95% CI, 1.1 to 23) and 5.6-fold (95% CI, 1.3 to 25) lower than that of the vector control, respectively; the peak VL values for the other cohorts, including the Gag-only cohort, were not statistically significantly different from that of the control cohort. In a previous study (4), the Gag-only vaccine also did not provide a meaningful reduction (only 40%) in peak VLs. The peak VLs of the Gag/Pol group were statistically lower than those of the Gag cohort (ratio of the geometric means = 4.0; 95% CI, 1.0 to 15.5); no other inter-vaccine-cohort differences in peak VLs were meaningful.

The post-acute phase of SIVmac239 infection was characterized by a plateau in VLs between 50 and 120 days (set point). The set point VL levels (taken as the geometric means

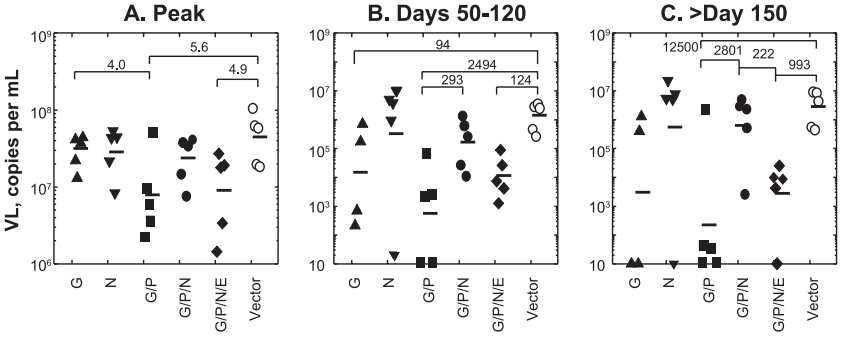

FIG. 4. Virological parameters for the vaccine and control cohorts. (A) Peak VLs. (B) Set point VLs. (C) Late-stage VLs. Shown are the VLs for the animals (individual symbols), as well as the geometric mean values (lines). Only those pairs of cohorts for which the differences were statistically significant are bracketed by horizontal lines in the upper portion of each panel. The ratios of the geometric mean values are indicated either beside or above the brackets. The individual animals were labeled according to the immunogens contained in the vaccine each received: G, Ad5/SIV Gag; N, Ad5/SIV Nef; G/P, Ad5/SIV Gag plus Ad5/SIV Pol; G/P/N, Ad5/SIV Gag plus Ad5/SIV Pol plus Ad5/SIV Nef; G/P/N/E, Ad5/SIV Gag plus Ad5/SIV Pol plus Ad5/SIV Nef plus Ad5/SIV Env.

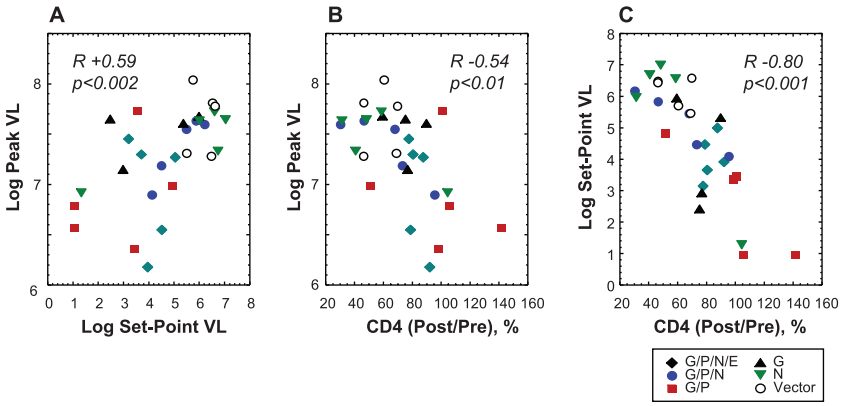

FIG. 5. Association of the peak VLs with set point VLs (A), peak VLs with the relative CD4 levels (B), and set-point VLs with the relative CD4 levels (C) for the individual animals. The relative CD4 levels are the ratios (in percentages) of set point CD4 levels (calculated as the geometric means of all the CD4 counts recorded between days 53 and 120 postchallenge) to the preinfection levels. The individual animals were labeled according to the immunogens contained in the vaccine each received: G, Ad5/SIV Gag; N, Ad5/SIV Nef; G/P, Ad5/SIV Gag plus Ad5/SIV Pol; G/P/N, Ad5/SIV Gag plus Ad5/SIV Pol plus Ad5/SIV Nef; G/P/N/E, Ad5/SIV Gag plus Ad5/SIV Pol plus Ad5/SIV Nef plus Ad5/SIV Env. The correlation coefficients of the Spearman rankings (for 24 vaccinees, excluding vector control animals) and the two-tailed *P* values are shown in each panel.

of VL data between days 53 and 122) are shown in Fig. 4B; only the values for the Gag, Gag/Pol, and Gag/Pol/Nef/Env cohorts were statistically lower than that of the control cohort. The only meaningful inter-vaccine cohort difference was between the set point VLs of the Gag/Pol and Gag/Pol/Nef cohorts (ratio = 293.0; 95% CI, 3.0 to 28,272.2). The effects on the set point VLs induced by any vaccine were, not surprisingly, more substantial (2,494-fold in the case of the Gag/Pol cohort relative to the control cohort) than that for the peak VLs (5.6-fold in the case of the Gag-Pol cohort). However, we found that the peak VLs determined the early set point levels on an individual animal basis ($P < 0.002$) (Fig. 5A).

A subset of vaccinees exhibited a rebound in VL from the initial set point level, while for a few, the VLs trended lower, even to the detection level of the assay. The cohort geometric means of the late-stage VLs (taken as the geometric mean of all VL data between days 178 and 360 for each macaque) for the Gag/Pol and Gag/Pol/Nef/Env groups were the only ones found to be statistically lower than that of the control cohort (Fig. 4C). In contrast to the results of our previous study, in which all 5 Ad5-Gag vaccine recipients had detectable viremia (4), 2 of 5 Gag-only vaccine recipients in the current study had undetectable VLs after 100 days postinfection.

Infection with SIVmac239 induced detectable declines in the circulating $CD4^+$ T-cell levels in macaques. The preservation of the peripheral CD4 T-cell levels correlated very well with the vaccine-elicited lowering of viremia measured either at the peak or at the set point (Fig. 5B and C).

Correlates of vaccine efficacy. The intracohort variabilities in the VLs were rather pronounced (Fig. 3), attesting to the inherent genetic variability of the outbred Indian rhesus macaques used in the study. There were one, two, and four of five Nef, Gag, and Gag/Pol vaccine recipients, respectively, that appeared to resolve their viremias. The single controller (animal 01D268) in the Nef cohort had the strongest vaccine-induced response (220 SFC/10^6 PBMCs at week 28) in the cohort. In contrast, the combination of Gag, Pol, and Nef immunogens did not produce a single long-term controller. The addition of Env immunogen to Gag/Pol/Nef vaccine led to a cohort performance with the lowest interanimal variability. It should be noted that only one macaque in the entire study (Gag/Pol/Nef/Env; 00C069) had the *B*08* allele, which was recently associated with control of SIV replication (23), but the presence of the *B*08* allele did not distinguish this animal from the rest of the cohort in terms of virus control.

Multiparametric correlation analyses of the prechallenge immune responses and the VLs were conducted using a nonparametric Spearman's ranking approach. These analyses were done in two ways: on an individual animal basis and on an individual cohort basis. No statistically meaningful correlation was found on a cohort-to-cohort basis; this was not unanticipated, given that the intracohort variabilities of the VL values for some groups approached the intercohort variability. In the study using the monovalent Gag vaccine, we showed that on an animal-to-animal basis, the viral load was correlated with the magnitude of the T-cell responses against the Gag CM9 epitope (4). For the current study, we asked (i) whether the addition of immune responses to other antigens would improve the association of the observed protective efficacy with the Gag-directed responses and (ii) which antigen-specific responses would do so. Each analysis excluded the control animals, as they would artificially bias the trend, and included only those animals that received at least one of the antigens for which the responses were to be correlated. For example, correlation analyses between the virological outcomes and the sum of the responses to Gag and Nef involved only Gag, Nef, Gag/Pol/Nef, and Gag/Pol/Nef/Env vaccine recipients.

Table 1 shows the correlation coefficients and *P* values for the various immune parameters measured at week 28 and the peak viral loads. Significant inverse correlations were observed between ELISPOT data collected at week 28 and the peak VLs and were improved when responses to other antigens were added to that of Gag. The correlations were comparable if the ELISPOT responses to the CM9 peptide were used instead of the response to the full Gag peptide pool (data not shown). However, the correlations were weaker when the prechallenge

TABLE 1. Rank correlations between prechallenge ELISPOT values and viral loads for macaques immunized with the monovalent or multivalent SIV vaccine[a]

ELISPOT (wk 28)	Peak VL			Set point VL		
	N	*R*	*P*	*N*	*R*	*P*
Gag	20	−0.37	0.11	19	−0.24	0.32
Gag + Nef	25	−0.47	0.02	24	−0.41	0.05
Gag + Pol	20	−0.49	0.03	19	−0.18	0.44
Gag + Pol + Nef	25	−0.49	0.02	24	−0.41	0.05
Gag + Pol + Env	20	−0.53	0.02	19	−0.15	0.52
Gag + Pol + Nef + Env	25	−0.54	0.01	24	−0.39	0.06

[a] Input immune variables are the sums of ELISPOT values (collected at week 28 or 4 weeks after dose 3) in SFC/10^6 PBMCs against the various antigens (Gag, Pol, Nef, and Env) collected at week 28 and week 34. The response variables are (i) peak VL calculated as the maximum VL value over the first month postchallenge and (ii) geometric means of VLs recorded over 50 to 123 days postinfection. *N*, number of animals used for each analysis; *R*, Spearman's rho correlation coefficient; *P*, two-tailed *P* value.

immunological parameters were analyzed against the set point VLs (Table 1). On a group-by-group basis, no statistically significant correlation could be established between the cohort geometric means of the VL values (peak or set point) and the cohort geometric means of the magnitude of the preinfection ELISPOT values.

On an animal-to-animal basis, no significant association was observed between the VLs (peak or set point) and the neutralization titers at day 14 or the geometric means of neutralizing titers taken over the period of days 45 to 150 postinfection ("set point" neutralization titers) for all vaccinees. The "set point" neutralization titers in the controllers were 3-fold higher than those of the noncontroller vaccinees, but the difference did not approach statistical significance. Also, the cohort geometric mean of the "set point" neutralizing titers for the Gag/Pol/Nef/Env group were highest among the vaccine cohorts but were only significantly different from those of the Nef group. This suggests that virus-specific neutralizing antibodies did not notably influence the course of viral infection among the vaccinees, although we cannot eliminate the possibility that the neutralizing activity was a covariate of the protective efficacy.

DISCUSSION

The study described here was designed to address whether multiantigen SIV vaccines (including the set of antigens homologous to that of the Step vaccine) delivered exclusively using a replication-defective adenovirus vector could be efficacious against the SIVmac239 challenge in terms of viral-load reduction and CD4 protection. The results (Table 1 and Fig. 4) show the benefit of antigen expansion beyond Gag for the virological outcome of the challenge; here, this effect was most consistent with the Gag/Pol and the Gag/Pol/Nef/Env cohorts. The benefits of antigen expansion were also observed in several other studies. The addition of heterologous Env antigens was shown to improve the efficacy of the Gag/Pol/Nef vaccine (20) and Gag-only vaccines (21) in animals lacking the *Mamu-A*01* allele against SHIV89.6P challenge. Wilson et al. (39) showed that the addition of Tat, Rev, and Nef to a Gag-only vaccine (4) improved the chronic control of SIVmac239 given intrarectally to mamu-A*01(+) monkeys; however, the caveat when making this comparison concerns the use of a repeated low-dose challenge with the multivalent vaccine as opposed to a high-dose challenge virus for the Gag-only-vaccinated macaques.

The trivalent Ad5 vaccine expressing HIV-1 clade B Gag, Pol, and Nef was shown to be highly immunogenic in phase I studies (29) and hence was considered the most promising vaccine modality in terms of eliciting HIV-1-specific cellular immunity in humans. The vaccine induced multifunctional HIV-1-specific CD4 and CD8 T cells in humans (7). Vaccine responses to each of the three antigen components were observed; the specificity and breadth of T-cell responses were more closely related to those observed during acute/early HIV-1 infection than to those in chronic infection (data not shown). The Step study showed that the trivalent HIV-1 vaccine lacked efficacy in terms of preventing acquisition or reducing viral loads in infected individuals (2). It also had the unexpected result that the vaccine may be associated with increased risk of HIV-1 acquisition in the participants, especially in uncircumcised males. In this paper, the SIV analog of the trivalent HIV-1 vaccine in the Step study was tested using *Mamu-A*01*$^+$ macaques and against a high-dose mucosal SIVmac239 challenge. The results using this vaccine trended toward an indication of limited efficacy, with 40% of the animals showing lowered VLs compared to any of the control animals; however, with the cohort size used in the study, the difference in VLs between the Gag/Pol/Nef and control cohorts was not statistically significant at any stage of the study (Fig. 4).

The better outcome for the Gag/Pol vaccine than for the Gag/Pol/Nef vaccine was a surprise for which we have no immediate scientific explanation. There was no sign of immunological interference by the addition of Nef to the vaccine. Kiepiela and coworkers (16) showed that the cellular responses to HIV-1 Nef in treatment-naïve infected individuals associated with increasing viremia and increased breadth of Gag-specfic immune responses was correlated with lower chronic viremia. In the same study, the Pol-specific responses did not influence viremia. The cellular responses to Nef after vaccination of macaques were less frequent than the responses to other immunogens. After virus challenge, the responses to all four immunogens increased substantially (data not shown); however, only the postchallenge responses to Gag were found to be correlated ($R = -0.59$; $P < 0.004$) with the set point VL. The postchallenge Nef responses did not appear to interfere with the ability to mount a vigorous response to Gag. Nef has many complex functions for promoting pathogenicity and immunoregulation, and inclusion in the vaccine may have detrimental effects associated with its activities (37). However, the Nef antigen used in the vaccine was deactivated by mutation of the myristoylation site glycine. Furthermore, *in vivo* expression using a replication-defective vector is expected to be highly limited. The Gag/Pol results described in this paper are indeed rather intriguing. It is unclear if the effects arose from other reasons, such as unaccounted-for host factors. As such, they warrant further verification with additional animal testing.

Several intrinsic differences and similarities between the animal challenge models and HIV-1 infection could determine the value of the preclinical models. The challenge model would represent the best-case scenario on the basis of two parame-

ters. First, the simian virus sequence used was often homologous to that of the vaccine strain. Such is rarely the case with HIV-1 infection, and for this reason, virus strains (e.g., SIVsmE660) that are heterologous to SIVmac239 have only recently been employed as challenge viruses (38). Second, the human testing of an HIV-1 vaccine is also complicated by the effects of highly variable vector preimmunity and host immunogenetic heterogeneity (3), which are more controlled in an animal study. Several SIV challenge studies involved macaques that had the *Mamu-A*01* allele because this allowed convenient monitoring of well-characterized CTL epitopes (4, 33). Recently, several investigators used monkeys without any of the known protective simian alleles (23, 27, 28), thereby adding to the genetic diversity of the animal cohorts. It should be noted that despite the fact that our study utilized *Mamu-A*01*$^+$ macaques, all 5 animals had substantially high VLs ($>10^5$ copies/ml), in contrast to 7 of 25 (28%) vaccine recipients showing no detectable VLs past 200 days postinfection; this is likely because none of these animals have any of the other protective alleles that would otherwise substantially improve their prognoses. Another notable difference between human and macaque infection models concerns the traditional use of high virus challenge doses in order to ensure 100% infection. In contrast, human exposure involves doses lower than the ones used for the macaque studies, and thus, one might assume that protection against a high-dose SIV challenge in macaques would be of higher stringency. Also, the use of high challenge doses would not have been able to detect any increased infection rates, as was observed in the Step study. To address this limitation, recent SIV challenge studies employed multiple low-dose mucosal challenges with SIV to establish infection, and this approach should mimic the clinically relevant human exposure to HIV (12, 38, 39).

Recently, several alternative immunization strategies and vectors have been reported in which the virological outcomes appeared to be improved over what we observed in this study and in a previous one (4) using an Ad5-based vaccine. A heterologous regimen using a recombinant Ad26-Gag prime followed by an Ad5-Gag boost afforded better control (1- and 2-log-unit better reduction in peak and set point VLs) of a SIVmac251 intravenously administered to *Mamu-A*01*$^-$/*B*17*$^-$ macaques than for the Ad5 prime/Ad5 boost control cohort (22). The outcome with the former regimen had been attributed to the higher magnitude, better breadth, and polyfunctionality of the induced T-cell responses. In another study, macaques immunized with a replicating rhesus cytomegalovirus (CMV) vector expressing SIV Gag, a Rev-Tat-Nef fusion, and Env exhibited resistance to infection at a rate of 40% by a repeated low-dose intrarectal challenge with SIVmac239 (12). This efficacy was associated with levels of effector memory CD8 and CD4 T cells higher than those induced using a nonreplicating vector, such as the recombinant Ad5 vaccine. In a third study, *Mamu-A*01*$^-$/*B*17*$^-$/*B*08*$^-$ macaques immunized using a DNA prime/Ad5 boost regimen with all SIV proteins except Env were able to resolve their viremia at a rate of 50% following a repeated low-dose intrarectal challenge with SIVsmE660 (38). These vaccine approaches appear to have promise on the basis of the challenge outcomes, especially relative to the analogous Step vaccine. Any future development of these alternative approaches will depend on (i) a better understanding of the shortcomings of the Ad5-based vaccine in the Step study and (ii) whether these alternative approaches can address such gaps appropriately.

Preclinical animal challenge models remain valuable tools in HIV-1 vaccine development. If carefully designed and sufficient in size, these tests can be instrumental in determining the relative efficacies of vaccine candidates; the trivalent vaccine analogous to the Step vaccine may represent a new benchmark for future testing of vaccine candidates. While it is understandable that the Step study results may have caused a heightened level of uncertainty about the future direction of HIV vaccine research, the opportunity for bridging our understanding of the value of preclinical tests to human clinical outcomes now presents itself.

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